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The structure of integument and wax glands of *Phenacoccus fraxinus* (Hemiptera: Coccoidea: Pseudococcidae)

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Abstract: Using scanning electron microscopy and optical microscopy, we studied the structure of the integument and wax glands of the mealybug, *Phenacoccus fraxinus* Tang (Hemiptera: Coccoidea: Pseudococcidae). We observed the ultrastructure of four wax pores including trilocular, quinquelocular, and multilocular pores as well as tubular ducts, recording characteristics of their structure, size and distribution. We found that that the integument of the mealybug consists of three main layers—the procuticle, epidermis and basement membrane—and four sub-layers of the procuticle—the epicuticle, exocuticle, endocuticle and formation zone. The wax-secreting gland cells were closely arranged in epidermis. All of them were complex and composed of one central cell and two or more lateral cells. These complex cells possess a large common reservoir for collection and storage. Synthesized by the glandular cells, the wax is excreted outside integument through canals.

Keywords: Pseudococcidae; *Phenacoccus fraxinus* Tang; Integument; Wax gland; Wax secretion

There are approximately 7,000 species of scale insects (Insecta: Hemiptera: Coccoidea) throughout the world, representing 20 families. Most are considered pests in agriculture, forests, fruit trees and ornamental plants and can be characterized by the special waxy covering on their body surface. Because of this protective wax, which protects against environmental factors and chemical pesticides, scale insects are hardy and often predominant, as compared to other insects (Ben-Dov & Hodgson, 1997). The protective wax coverings are formed by the wax substances secreted from the various wax pores that arise out of the integument. The wax pores and their glands are well-developed in scale insects, and possess diversity in both type and structure.

The unique features of the wax covering make the integument of scale insects differ from other insects in many ways. This difference makes it significant to study the structure and function of the integument of scale insects, but reports on this subject are scarce (Foldi, 1985, 1995; Bielenin & Weglarska, 1990, 1992; Waku & Manabe, 1981). In China, early research focused on the white wax insect, *Ericerus pela* Chavannes (Zhang et al, 1988; Tan & Zhang, 1992). Likewise a few studies reported the ultrastructure and wax-secretion processes in family Pseudococcidae (Cox & Pearce, 1983; Kuma, 1997; Jansen, 2001). We hope to enrich these findings by obtaining a comprehensive view of the integument's ultrastructure and associated wax glands and wax secretions of the mealybug, *Phenacoccus fraxinus* Tang (Family: Pseudococcidae) using standard histological examination of serial sections and scanning electron microscopy (SEM).

MATERIAL AND METHODS

Dissections of insects and light microscope observation

Live scale insects were collected from their host plant, *Fraxinus chinensis* Roxb, in Taiyuan, Shanxi Province, China, from April to May, 2007. These insects were kept alive and mounted individually on a wax dish using #00 sized insect pins. Each insect was then dipped in physiological saline (0.9%, v/v) to ensure preservation of the integument. Using a magnifying lens, the dorsum of each insect was dissected and the integument structures were removed and placed in glutaraldehyde (0.5%, v/v).

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In total, 30 integument structures were used both for serial section and SEM.

For basic morphological examinations, whole specimens were mounted on glass slides using methods as described by Kosztarab (1967). Twenty adult females were examined with light microscopy at 10× and 40×

Serial sections of integument

Fifteen integument structures were embedded in paraffin by first dehydrating the tissues in a series of increasing concentrations of ethyl alcohol (10 min each in 35, 55, 75, 85, 95, and 100% [v/v]) and xylene (10 min each in 35, 55, 75, 85, 95, and 100% [v/v]). The tissues were then immersed in an equal volume mixture of xylene:paraffin for 48 h at 56 ℃ and then in paraffin for 48 h at 56 ℃ before embedding in paraffin. Embedded specimens were serially sectioned to a thickness of 0.6 μm and immersed in 100% xylene and then decreasing concentrations of ethyl alcohol (10 min each in 100, 95, 85, 75, 55, and 35% [v/v]) followed by a rinse with distilled water (10 min). These were then stained with 2 % ferrovanadium (30 min) and hematoxylin (1.5 h) and intermediately rinsed in distilled water (10 min). Stained sections were rinsed in flowing water (10 min), immersed in picric acid (1.5 h), rinsed again in flowing water (30 min) and immersed in hartshorn (1:100), after which they were dehydrated through a series of increasing concentrations of ethyl alcohol (10 min each in 35，55，75，85，95，and 100% [v/v]). Xylene was used as a mounting medium. Integument sections were observed and photographed with optic microscopy (Olympus BX51, Olympus Optical Co., Japan).

Scanning electron microscopy (SEM)

For SEM study, 15 adult females were fixed in 4% glutaraldehyde for 48 h, rinsed three times in phosphate buffer (0.2 mol/L) and then dehydrated through successive 5 min changes in 70, 80, 90, 100% ethanol[v/v] and three further changes in 100%. The ethanol was then displaced by liquid carbon dioxide and samples were dried using EMS 850 critical point dryer, mounted on copper stubs, then coated with gold in the sputter coater and finally scanned at different angles using the SEM (JSM-35C, JEOL, Japan) at 25 kv.

RESULTS

The wax pores and wax secretion

The body of adult female, *P. fraxinus* was oval in shape, with distinct segments, approximately 6.0 mm long and 3.6 mm wide. The surface of the body was covered with a thin layer of white wax substances, but with less wax on the intersegmental folds (Figure 1a). During oviposition, pregnant adult females secreted an ovisac twice as long as their body deposited eggs in the ovisac.

By SEM, the ultrastructure of the wax pores and their wax secretions was observed. Trilocular disc pores, the first type of the wax pores, were abundant and spaced evenly over both the dorsum and venter but were absent in intersegmental areas. Each of the trilocular pores, with a diameter of about 4.5 μm, had three narrow 8-shaped openings 2 μm long and 1 μm wide arranged in a spiral shape. Each opening secreted a flat shape wax filament 2 μm in width (Figure 1b). These wax filaments linked on the surface of the body, forming the wax covering. Along the dorsal margin, 18 pairs of cerarii were arranged in segments from the anterior end to the last on the anal lobes. A cerarius from which the lateral wax filament normally arose consisted of two or more conical or lanceolate setae accompanied by a cluster of trilocular pores (Figure 1c). Each anal lobe cerarius had five enlarged conical or lanceolate setae accompanied by 11 trilocular pores in a cluster on a sclerotised area. There were two conical setae and four trilocular pores in cerarii pairs 16 and 17, three to four conical setae and three trilocular pores in pair 3, two conical setae and three trilocular pores in pairs 12 through 15, and two conical setae and two trilocular pores in other pairs.

Quinquelocular pores, the second type of wax-secreting pores, were generally restricted to the stigmatic furrow on the ventral surface. Each quinquelocular pore, 6 μm in diameter with a 2 μm-wide rim, consisted of five circular loculi or micro-orifices, arranged in a circle or pentagon with each loculus further secreting one wax filament, 0.9-1.0 μm in diameter (Figure 1d). Multilocular pores, the third type, were found ventrally associated with the area around the vulva. Each multilocular pore normally had 10-12 circular loculi arranged in a circle (Figure 1e) and each loculus secreted one fine wax filament, 0.9 μm in diameter. These filaments were broken into short and curved fragments after being secreted from the pore and conglutinated on the surface of eggs, both to prevent them from sticking and to protect them from immersion and desiccation. (Figure 1f). Tubular ducts, the fourth type of the wax-secreting pores, had a dense distribution on the dorsal abdominal segments, the thoracic margin and submargin, but absent in middle area of the head. On ventral surface, tubular ducts consisting of an outer ductile of 18 μm in length and 4 μm in diameter and an inner ductile of 6 μm in length and 1.5 μm in diameter (Figure 1g) were scattered over most of the thoracic and abdominal segments. Each outer opening of the tubular ducts on the cuticular surface was a round pore, 4 μm in diameter, from which a long, hollow 2 μm in diameter wax filament was secreted. This type of filament seems to be used as a frame in cocoon and ovisac construction (Figure 1h). By optical microscopy, we saw and documented four main types of wax pores were seen on the cuticle of mounted specimens (Figure 2).

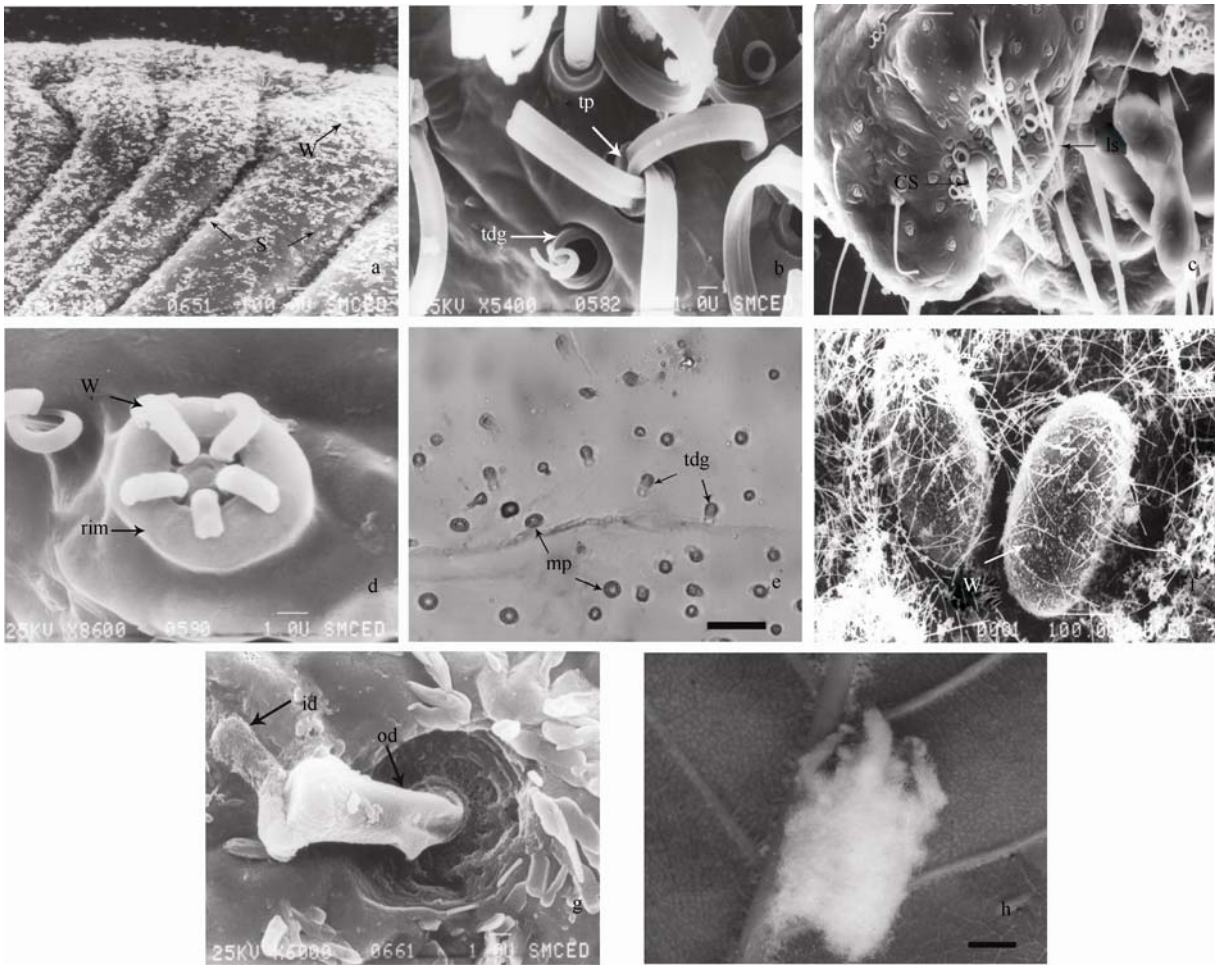

Figure 1 The SEM photographs of the different structures of *Phenacoccus fraxinus*

a) SEM photograph of part of the dorsal surface of the adult female, showing wax (W) covering on segments (S) but much less wax on intersegmental area; magnification 60×, bar=100 μm; b) SEM photograph showing a trilocular pore (tp) and tubular duct gland (tdg); magnification 5 400×, bar=1 μm; c) SEM photograph showing a cerarius consisting of two thick conical and 5-8 lanceolate setae (cs and ls) and accompanied by many trilocular pores in cluster; magnification 1 000×, bar=10 μm; d) SEM photograph of a quinquelocular pore with a rim and five round loculi arranged in a pentagon shape, and each loculi secreting a wax filament (w); magnification 8 600×, bar=1 μm; e) Micrograph of multilocular pores (mp) and tubular duct glands (tdg) in the integument of *Phenacoccus fraxinus*; magnification 600×, bar=50 μm; f) SEM photograph of eggs in the ovisac of *Phenacoccus fraxinus*, showing surface of eggs conglutinated with a lot of fine wax granule (W) secreted by multilocular pores; magnification 200×, bar=100 μm; g) SEM photograph of a tubular duct inside the integument showing outer and inner ductiles (od and id); magnification 6 000×, bar=1 μm; h) Dorsal view of ovisac showing wax all over the body except prothorax; magnification 10×, bar=1 mm.

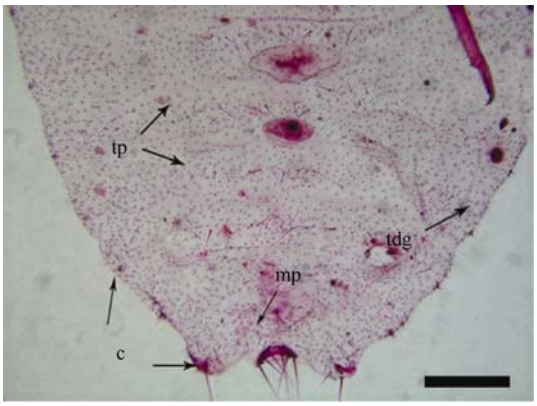

Figure 2 Micrograph of the mounted specimens of the adult female, *Phenacoccus fraxinus* showing the density of wax-secreting pores over the integument and cerarii (c)

magnification150×, bar=200 μm

Integument structure and wax glands

Viewing serial sections of integument tissues through a light microscope revealed that the integument of *P. fraxinus* consisted of three main layers: the procuticle, epidermis and basement membrane (Figure 3). The procuticle was the outermost integument layer, between 4.2 and 6.7 μm in thickness, containing a further four sub-layers: the epicuticle, exocuticle, endocuticle and formation zone. The epidermis was beneath the formation zone, being only a single layer of cells. All of wax-secreting glands in scale insects' integument were arranged closely, one-by-one in the epidermis, and were particularly well developed. The cell of each wax-secreting gland was composed of one central cell and two or more lateral cells, organized into the complex cell of the wax-secreting gland. This structure is what allows

for the glands' relatively large size and apple-like shape. More specifically, the wax gland complex cell of the trilocular pore contained one central cell and two lateral cells, with some wax-like material in the central section of cell where the real wax was secreted. Meanwhile, the complex cell of each quinquelocular pore was composed of five identical glandular cells cuddling together (Figure 4), each opening into a large common reservoir beneath the actual pore. Synthesized by the glandular cells, this secretion was collected and stored in the common reservoir from which wax was extruded via the short tube, leading to the actual pore of each loculus. Similarly, the complex cell for multilocular pores consisted of between six and twelve tightly folded glandular cells. Structurally, the orifices of multilocular pores possessed the same characteristics as described for the quinquelocular pore glands. The wax-secreting gland of each tubular duct, however, consisted of a large central glandular cell at the end of an inner ductile, surrounded by four to six lateral glandular cells attached to the inner end of the outer ductile (Figure 5). The central cell had a reservoir in which wax substances were contained as well as an irregularly-shaped receptor ductile from which the wax could be secreted.

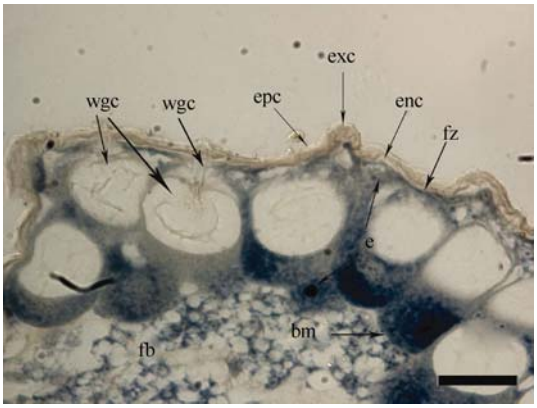

Figure 3 Micrograph of the integument in the adult female, Phenacoccus fraxinus showing the procuticle, epidermis (e) and basement membrane (bm)

Four layers of procuticle i.e. epicuticle (epc), exocuticle (exc), endocuticle (enc) and formation zone (fz) and a layer of wax gland cells (wgc) arranged closely one after the other in the epidermis (e) of the integument of the insect are also visible. Behind the epidermis is present a fat body, primarily originated from the wax material secreted by wax gland tube (wgt). magnification 600×, bar=75 μm

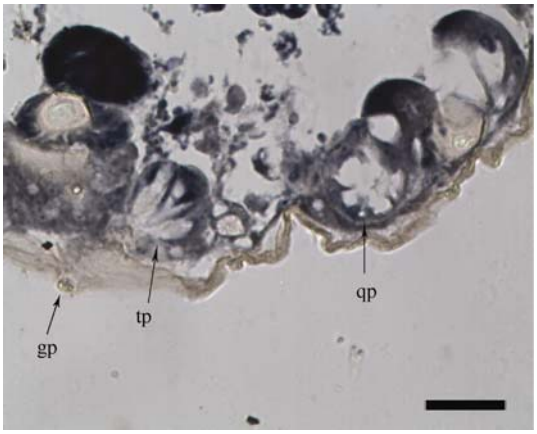

Figure 4 Micrograph of gland pores (gp), trilocular pore (tp) and quinquelocular disc-pore (qp) in the integument of Phenacoccus fraxinus

magnification 600×, bar = 75 μm

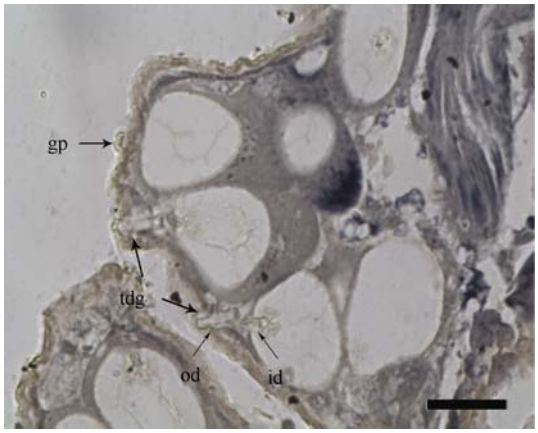

Figure 5 Micrograph of gland pores (gp) and tubular duct glands (tdg) (including inner ductile (id) and outer ductile (od)) in integument of *Phenacoccus fraxinus*

magnification 600×, bar = 75 μm

Beneath the basement membrane, a large amount of fat bodies were found suspended in the haemocoele. The granules of the fat body were about 1 μm in diameter.

DISCUSSION

The insects' integument is a complicated tissue enwrapping the whole body, including the legs which came into the ectoskeleton and offered attachment and support for the endoskeleton (Chapman, 1998). Consequently, the integument conformation dominated the build and exterior characters of the insect. The integument is an important protective tissue that prevents inner moisture from excessive evaporation and environmental attacks via inorganic compounds, pathogen infections, microorganisms, and insecticide sprays, etc. (Gullan & Cranston, 2005). In comparison with other insects, the mealybug, *P. fraxinus* has evolved its integument with a strong functional structure. A great deal of wax-secreting gland cells were densely arranged in the epidermis, a 19.6 μm（16.3-23.8 μm）thick layer, the thickest of the integument.

Consisting of trilocular pores, quinquelocular pores, multilocular pores and tubular duct glands, the wax-secreting gland cells were large, complex cells capable of synthesizing waxy substances using raw materials from

the haemolymph in the coelom. The wax substances were collected and stored in the common reservoir then transferred to the outer surface of integument through the wax pore canals. This complicated process to deal with wax in the integument is unique. Due to the well developed wax-secreting glands in the integument layers, the body surface of the scale insects remains well protected by the wax covering secreted from the wax pores.

In our study, trilocular pores and tubular ducts were the major types of the wax-secreting pores in the integument of *P. fraxinus*. Based on our observation of the wax-secretion process of the mealybug, the trilocular pores were fairly numerous and wax filaments that were secreted to form the wax covering on the mealybug surface in all instars and similar wax was present on the lateral filaments formed from the cerarii. In contrast, the wax substances were not formed in tubular ducts until the adult females entered oviposition and their wax filaments were thick and hollow, to frame the ovisac.

Trilocular pores and tubular ducts in integument are also present in other scale insects, such as *Ceroplastes japonicus* Green, a species of family Coccidae (Xie et al, 2006). However, the trilocular pores only occurred on the dorsal surface in the third instar of female nymphs and adults. The three round loculi of the trilocular pores were arranged in a triangle or parallel, in which soft wax substances were formed containing honeydew, named "wet wax," which formed the protective coating over the body of the scales. Conversely, tubular ducts were arranged in a submargin band on the ventral surface in the third instar of female nymphs and adult of *C. japonicus*. Secreted from these tubular ducts, the wax was very fine wax filaments that broke into small fragments or powder, covering the ventral surface and thus playing a protective role. These characteristics of the trilocular pores and tubular ducts with their wax secretions are absolutely different from those in the mealybug, *P. fraxinus*. The process of staggered wax-secretion of the tubular ducts can be divided into two phases, one in late autumn and secreted by the older second instar nymphs to produce the cocoons for the mealybugs living through the winter, and the other in early summer and secreted by adult females to make the ovisac. In spite of their presence in other stages, tubular ducts did were not participate in the process of wax secretion.

During the course of this study, we observed many globoid fat bodies beneath the basement membrane of the integument of *P. fraxinus*. These fat bodies may possibly be primarily originated from the wax synthesized in the wax-secreting glands. However, our current knowledge about this mechanism of wax synthesis is incomplete and requires further research.

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